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1. Your reference — 02157

2. Patent application number *(The Patent Office will fill in this part)* — **0205347.8** — 7 MAR 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

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Patents ADP number *(if you know it)*

7728552002

7728560002

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

Biologically Active Complex

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Patents ADP number *(if you know it)*

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Biologically Active Complex

The present invention relates to biologically active complexes, in particular complexes derived from alpha-lactalbumin, to pharmaceutical compositions containing these as well as to their use in therapy, in particular as anti-cancer or antibacterial agents.

Biologically active complexes obtained from milk and particularly human milk, together with their use as antibacterial agents is described for example in EP-0776214.

HAMLET (formerly known as MAL) is a molecular complex that induces *in vitro* apoptosis selectively in tumour cells, but not in healthy differentiated cells. The apoptotic activity of this variant fold was discovered by serendipity, in a fraction of human milk casein obtained by precipitation at low pH, and was purified by ion exchange chromatography, eluting as a single peak after 1M NaCl. The elute was shown by spectroscopy to contain partially unfolded α-lactalbumin in an apo-like conformation (M. Svensson, et al, (1999) *J Biol Chem,* **274**, 6388-96), with native-like secondary structure, but lacking specific tertiary packing of the side chains. The link between apoptosis induction and the folding change was proven by deliberate conversion of native α-lactalbumin to the apoptosis inducing form (M. Svensson, et al., (2000) *Proc Natl Acad Sci* USA, **97**, 4221-6). HAMLET was shown to bind to the surface of tumour cells, to translocate into the cytoplasm and to accumulate in cell nuclei, where it causes DNA fragmentation (M. Svensson, et al., (2000) *Proc Natl Acad Sci* USA, **97**, 4221-6).

It has also been found that other reagents and specifically lipid such as oleic acid, are useful in the conversion of human α-lactalbumin to HAMLET (human α-lactalbumin made lethal to tumour cells). In particular, it has been reported previously that

oleic acid (C18:1:9cis) is required for HAMLET production (M. Svensson, et al., (2000) *Proc Natl Acad Sci* USA, **97**, 4221-6).

α-Lactalbumin is a 14.2 kDa globular protein with four α-helices (residues 1-34, 86-123) and an anti-parallel β-sheet (residues 38-82), linked by four disulphide bonds (61-77; 73-91; 28-111 and 6-120) (K. R. Acharya, et al., (1991) *J Mol Biol*, **221**, 571-81). The native conformation of α-lactalbumin is defined by a high affinity Ca^{2+} binding site, co-ordinated by the side chain carboxylates of Asp82, Asp87 and Asp88, the carbonyl oxygens of Lys79 and Asp84, and two water molecules (K. R. Acharya, et al., (1991) *J Mol Biol*, **221**, 571-81). The protein adopts the so called apo-conformation found in HAMLET when exposed to low pH, or in the presence of chelators, that release the strongly bound Ca^{2+} ion (D. A. Dolgikh, et al., (1981) *FEBS Lett*, **136**, 311-5; K. Kuwajima, (1996) *Faseb J*, **10**, 102-09).

The applicants have found that in order to achieve HAMLET with apoptotic activity, requires both a conformational or folding change and the presence of a lipid cofactor and this may preferably be achieved using a variant of alpha-lactalbumin. Furthermore, they have found that the optimal cofactors for the conversion of alphalactalbumin to HAMLET are C18:1 fatty acids with a double bond in the cis conformation at position 9 or 11. Saturated C18 fatty acid or unsaturated fatty acids in the trans conformation, or fatty acids with shorter carbon chains could not form HAMLET, suggesting that highly specific inter-molecular interactions are required for lipids to act as folding partners in this system.

According to the present invention there is provided a biologically active complex comprising
(i) a cis C18:1:9 or C18:1:11 fatty acid or a different fatty acid with a similar configuration; and

been released or which does not have a functional calcium binding site; or a fragment of either of any of these, provided that any fragment comprises a region corresponding to the region of α-lactalbumin which forms the interface between the alpha and beta domains, and further provided that when (ii) is alphalactalbumin, (i) is other than C18:1:9 cis fatty acid.

As used herein the expression "variant" refers to polypeptides or proteins which are homologous to the basic protein, which is suitably human or bovine α-lactalbumin, but which differ from the base sequence from which they are derived in that one or more amino acids within the sequence are substituted for other amino acids. Amino acid substitutions may be regarded as "conservative" where an amino acid is replaced with a different amino acid with broadly similar properties. Non-conservative substitutions are where amino acids are replaced with amino acids of a different type. Broadly speaking, fewer non-conservative substitutions will be possible without altering the biological activity of the polypeptide. Suitably variants will be at least 60% identical, preferably at least 70%, even more preferably 80% or 85% and, especially preferred are 90%, 95% or 98% or more identity.

Identity is suitably measured using any of the known algorithms which determine similarity or homology, in particular, the

In a particularly preferred embodiment, the variant used in the method of the invention is one in which the calcium binding site has been modified so that the affinity for calcium is reduced, or it is no longer functional. It has been found that in bovine α-lactalbumin, the calcium binding site is coordinated by the residues K79, D82, D84, D87 and D88. Thus modification of this site, for example by removing one of more of the acidic residues, can reduce the affinity of the site for calcium, or eliminate the function completely and mutants of this type are a preferred aspect of the invention.

The Ca^{2+}-binding site of bovine α-lactalbumin consists of a 3_{10} helix and an α-helix with a short turn region separating the two helices (Acharya K. R., et al., (1991) *J Mol Biol* **221**, 571-581). It is flanked by two disulfide bridges making this part of the molecule fairly inflexible. Five of the seven oxygen groups that co-ordinate the Ca^{2+} are contributed by the side chain carboxylates of Asp82, 87 and 88 or carbonyl oxygen's of Lys79 and Asp84. Two water molecules supply the remaining two oxygen's (Acharya K. R., et al., (1991) *J Mol Biol* **221**, 571-581).

Site directed mutagenesis of the aspartic acid at position 87 to alanine (D87A) has previously been shown to inactivate the strong calcium-binding site (Anderson P. J., et al., (1997) *Biochemistry* **36**, 11648-11654) (Fig. 1a) and the mutant proteins adopted to the apo- conformation.

Therefore in a particular embodiment, the aspartic acid residue at amino acid position 87 within the protein sequence is mutated to a non-acidic residue, and in particular a non-polar or uncharged polar side chain.

Non-polar side chains include alanine, glycine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan or cysteine. A particularly preferred examples is alanine.

Uncharged polar side chains include asparagine, glutamine, serine, threonine or tyrosine.

In order to minimize the structural distortion in the mutant protein, D87 has also been replaced by an asparagine (N) (Permyakov S. E., et al., (2001) *Proteins Eng* **14**, 785-789), which lacks the non-compensated negative charge of a carboxylate group, but has the same side chain volume and geometry (Fig. 7a). The mutant protein (D87N) was shown to bind calcium with low affinity

Such a mutant forms a further preferred embodiment of the invention.

Thus particularly preferred variants for use in the complexes of the invention are D87A and D87N variants of α-lactalbumin, or fragments which include this mutation.

When comparing amino acid sequences for the purposes of determining the degree of identity, programs such as BESTFIT and GAP (both from Wisconsin Genetics Computer Group (GCG) software package). BESTFIT, for example, compares two sequences and produces an optimal alignment of the most similar segments. GAP enables sequences to be aligned along their whole length and finds the optimal alignment by inserting spaces in either sequence as appropriate. Suitably, in the context of the present invention when discussing identity of sequences, the comparison is made by alignment of the sequences along their whole length.

The term "fragment thereof" refers to any portion of the given amino acid sequence which will form a complex with the similar activity to complexes including the complete α-lactalbumin amino acid sequence. Fragment may comprise more than one portion from within the full length protein, joined together. Portions will suitably comprise at least 5 and preferably at least 10 consecutive amino acids from the basic sequence.

Suitable fragments will be deletion mutants suitably comprise at least 20 amino acids, and more preferably at least 100 amino acids in length. They include small regions from the protein or combinations of these.

The region which forms the interface between the alpha and beta domains is, in human α-lactalbumin, defined by amino acids 34-38 and 82-86 in the structure. Thus suitable fragments will include these regions, and preferably the entire region from amino acid 34-86 of the native protein.

This region of the molecule differs between the bovine and the human proteins, in that one of the three basic amino acids (R70) is changed to S70 in bovine α-lactalbumin thus eliminating one co-ordinating side chain. It may be preferable therefore, that where the bovine α-lactalbumin is used in the complex of the invention, an S70R mutant is used.

It appears that three molecular events are required to form HAMLET from α-lactalbumin. First, the tightly bound Ca^{2+}-ion is released. The apo-protein is then allowed to bind the lipid cofactor, for example, on an ion exchange matrix. Third, the active complex is eluted at high salt and dialysed. The elutes were characterised after repeating this procedure with 14 closely related fatty acids as shown hereinafter. Only the C18:1:9cis and C18:1:11cis complexes were found to cause apoptosis, and they alone gave distinct novel signals by NMR, indicating that they formed a novel molecular complex. Several other fatty acids were capable of retaining the protein on ion exchange matrices and to stabilize the protein in a partially unfolded conformation, but they did not form biologically active complexes and gave sharper NMR signals as expected from a mixture of protein and fatty acid. It appears that the unsaturated C18:1cis fatty acids have unique structural features, allowing them to form HAMLET from apo-α-lactalbumin, and suggest that they differ from the other fatty acids in that they offer the correct stereo-specific match. The lack of significant HAMLET formation with a number of closely related fatty acids suggests a highly selective and specific process. Consequently, any other fatty acids used in the complex of the invention should have essentially similar stereospecificity to these unsaturated C18:1cis fatty acids.

The Ca^{2+} binding site is 100% conserved in α-lactalbumin from different species (Acharya K. R., et al., (1991) *J Mol Biol* 221, 571-581) illustrating the importance of this function for the protein.

with D88 initially dock the calcium ion into the cation-binding region, and form internal hydrogen bonds that stabilise the structure (Anderson P. J., et al., (1997) *Biochemistry* **36**, 11648-11654). A loss of either D87 or D88 has been shown to impair Ca2+ binding, and to render the molecule stable in the partially unfolded state (Anderson P. J., et al., (1997) *Biochemistry* **36**, 11648-11654).

Further, as reported hereinafter, mutant proteins with two different point mutations in the calcium-binding site of bovine α-lactalbumin were used. Substitution of the aspartic acid at position 87 by an alanine (D87A) totally abolished calcium binding and disrupted the tertiary structure. After substitution of the aspartic acid by asparagine, the protein (D87N) still bound calcium but with lower affinity and showed a loss of tertiary structure, although not as pronounced as for the D87A mutant (Permyakov S. E., et al., (2001) *Proteins Eng* **14**, 785-789). The mutant protein showed a minimal change in packing volume as both amino acids have the same average volume of 125Å^3, and the carboxylate side chain of asparagines allow the protein to co-ordinate calcium, but less efficiently (Permyakov S. E., et al., (2001) *Proteins Eng* **14**, 785-789). Both mutant proteins were stable in the apo-conformation at physiologic temperatures but despite this conformational change they were biologically inactive in the apoptosis assay. The results demonstrate that a conformational change to the apo-conformation alone is not sufficient to induce apoptosis.

The structure of α-lactalbumin is known in the art, and the precise amino acid numbering of the residues referred to herein can be identified by reference to the structures shown for example in Anderson et al. supra. and Permyakov et al supra.

Native bovine α-lactalbumin and the Ca^{2+} mutants could be converted to the HAMLET like complex named BAMLET, showing that the same fatty acid stabilised bovine α-lactalbumin in the BAMLET

conformation. The conversion yield was lower, however, suggesting that lipid binding to the bovine protein was less efficient. The structural basis for this effect is not clear. Bovine and human α-lactalbumin show 76% amino acid sequence identity and have similar native conformations (Wijesinha-Bettoni R., et al., (2001) *J Mol Biol* **312**, 261-273). The lower conversion yield for BAMLET suggested that the sequence differences influenced the fit between the fatty acid and the bovine protein. The divergent sequences are mainly located in the α-helical region (A-helix 57%, B-helix 50%, C-helix 23% and 3_{10}-helix 25% difference) but this region is unlikely to be involved in fatty acid binding (see Example 3 above).

Our results demonstrate that the change in biologic function requires not just a conformational change of the protein, but also the lipid cofactor. The dual requirements for a change in protein conformation and a lipid cofactor may be important to achieve tissue specificity. The active complex should only be formed in local environments that favor the altered protein fold, and where lipid cofactors are available. In the case of HAMLET, such conditions are present in the stomach of the breast-fed child. The low pH precipitates casein with α-lactalbumin in the apo-conformation, and activates pH sensitive lipases that release oleic acid from the milk phospholipids. It is interesting to note that α-lactalbumin and oleic acid respectively, are the most abundant proteins and fatty acid in human milk. The lipids thus appear to function as "post-secretion chaperones", involved in the adaptation of proteins to shifting external environment. The need for both a folding change and a tissue specific lipid makes sense in order to protect tissues from the occasional protein folding variant on the loose, and to target the site where the novel function is needed.

Complexes of the invention are useful in a variety of therapeutic

tract. For these purposes, the complex is suitably formulated as a pharmaceutical composition and these form a further aspect of the invention.

The complex can be administered in the form of an oral mucosal dosage unit, an injectable composition, or a topical composition. In any case the protein is normally administered together with the commonly known carriers, fillers and/or expedients, which are pharmaceutically acceptable.

In case the protein is administered in the form of a solution or cream for topical use the solution contains an emulsifying agent for the protein complex together with a diluent or cream base. Such formulations can be applied directly to the tumour, or can be inhaled in the form of a mist into the upper respiratory airways.

In oral use the protein is normally administered together with a carrier, which may be a solid, semi-solid or liquid diluent or a capsule. Usually the amount of active compound is between 0.1 to 99% by weight of the preparation, preferably between 0.5 to 20% by weight in preparations for injection and between 2 and 50% by weight in preparations for oral administration.

In pharmaceutical preparations containing complex in the form of dosage units for oral administration the compound may be mixed with a solid, pulverulent carrier, as e.g. with lactose, saccharose, sorbitol, mannitol, starch, such as potato starch, corn starch, amylopectin, cellulose derivatives or gelatine, as well as with an antifriction agent, such as magnesium stearate, calcium stearate, polyethylene glycol waxes or the like, and be pressed into tablets. Multiple-unit-dosage granules can be prepared as well. Tablets and granules of the above cores can be coated with concentrated solutions of sugar, etc. The cores can also be coated with polymers which change the dissolution rate in the gastrointestinal tract, such as anionic polymers having a pk_a

of above 5.5. Such polymers are hydroxypropylmethyl cellulose phthalate, cellulose acetate phthalate, and polymers sold under the trade mark Eudragit S100 and L100.

In preparation of gelatine capsules these can be soft or hard. In the former case the active compound is mixed with oil, and the latter case the multiple-unit-dosage granules are filled therein.

Liquid preparations for oral administration can be present in the form of syrups or suspensions, e.g., solutions containing from about 0.2% by weight to about 20% by weight of the active compound disclosed, and glycerol and propylene glycol. If desired, such preparations can contain colouring agents, flavouring agents, saccharine, and carboxymethyl cellulose as a thickening agent.

The daily dose of the active compound varies and is dependant on the type of administrative route, but as a general rule it is 1 to 100 mg/dose of active compound at personal administration, and 2 to 200 mg/dose in topical administration. The number of applications per 24 hours depend of the administration route, but may vary, e.g. in the case of a topical application in the nose from 3 to 8 times per 24 hours, i.e., depending on the flow of phlegm produced by the body treated in therapeutic use.

The invention further provides a method for treating cancer which comprises administering to cancer cells a complex or a composition as described above.

The invention further provides a method for treating bacterial infections which comprises administering to a patient in need thereof, a complex or a composition as described above.

In the description, the following abbreviations have been used.

EDTA: ethylenediamintetra acetic acid, Tris tris(hydroxymethyl)aminomethane, ANS: 8-Anilinonaphtalene-1-sulfonic acid,
CD: circular dichroism,
UV: ultra violet,
NaCl: sodium chloride,
NMR: nuclear magnetic resonance, ppm: parts per million
FITC: fluorescein isothiocyanate,
TLC: thin layer chromatography,
DEAE: diethylaminoethyl,
HCl: hydrochloric acid,
EGTA: ethylene-bis(oxyethyleneitriol)tetraacetic acid.
FPLC; fast protein liquid chromatography;
PBS, phosphate-buffered saline.

The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings in which:

Figure 1 shows simplified fatty acid structures and in particular line drawings of the unsaturated fatty acids, which were investigated for their ability to produce a HAMLET like molecular complex. C16:1:9cis = Palmitoleic acid, C18:1:6cis = Petroselinic acid, C18:1:9cis = Oleic acid, C18:1:11cis = vaccine acid, C20:1:11cis = Eicosenic acid, C18:1:9trans = Elaidic acid, C18:1:11trans = Trans vaccenic acid, C20:4,5,8,11,15cis = Arachidonic acid, C18:3:6,9,12cis = Gamma linolenic acid, C18:3:9,12,15cis = Linolenic acid, C18:2:9,12cis = Linolenic acid.

Figure 2 is a series of graphs showing the retention of apo- α-lactalbumin on ion exchange matrices conditioned with individual fatty acids.

Figure 3 illustrates tumour cell apoptosis induced by the lipid-protein complexes.

Figure 4 shows the results of CD spectroscopy to determine the tertiary structure of the fatty acid-protein complexes.

Figure 5 shows the results of probing of the fatty acid-protein complexes by ANS spectroscopy as an indicator of hydrophobicity.

Figure 6 shows the results of NMR analysis of complexes.

Figure 7 illustrates the characterisation of the D87A and D87N mutants of α-lactalbumin, in which panel A shows the structure of the calcium-binding site.

Figure 8 illustrates biological tests carried out using mutated proteins alone, and shows that they do not induce apoptosis.

Figure 9 illustrates the conversion of bovine α-lactalbumin to BAMLET, where panel A shows elution peaks obtained during the preparation, panels B and C relate to the biological testing of BAMLET, panel D shows the results of near UV CD spectroscopy, panel E shows the results of intrinsic fluorescence spectrometry, and panel F shows the ANS spectra of HAMLET and BAMLET.

Figure 10 illustrates the production and test results for D87A and D87N to D87A-and D87N-BAMLET, where panel A shows elution peaks obtained during the preparation, panels B and C relate to the biological testing of these complexes, panel D shows the results of near UV CD spectroscopy, panel E shows the results of intrinsic fluorescence spectrometry, and panel F shows the ANS spectra.

Example 1

Structural variants of oleic acid, and other fatty acids differing in the degree of saturation, carbon chain length and cis/trans conformation (Fig. 1) were compared for their ability

Apo α-lactalbumin was applied to column matrices that had been pre-conditioned with each indicated fatty acid indicated in Figure 1 using the method described by M. Svensson, et al., (2000) *Proc Natl Acad Sci* USA, **97**, 4221-6, and the eluate after 1M NaCl was collected. Columns conditioned with C18:1:9cis were used as a positive control (a). All unsaturated fatty acids in the cis conformation retained apo-α-lactalbumin on the column, but with varying efficiency. Unsaturated fatty acids in the trans conformation (C18:1, 9trans, C16:1, 9trans) or saturated fatty acids (C6:0 and C18:0) failed to retain apo-α-lactalbumin on the column.

Results of ion exchange chromatography on fatty acid conditioned matrices are shown in Fig. 2. Unsaturated C18 fatty acids in the cis conformation formed complexes with apo-α-lactalbumin. The C18:1:9cis fatty acid converted > 90% of the added apo-α-lactalbumin. The C18:1:11cis fatty acid was somewhat less efficient with a yield of about 70%, and other unsaturated C18 cis fatty acids (C18:1:6cis, C18:2:9,12cis, C18:3:9,12,15cis and gamma C18:3:6,9,12cis gave considerably lower yields.

Trans conformers of C18:1 and saturated fatty acids were practically inactive, however. Only small amounts of protein eluted with high salt from columns conditioned with C18:1:9trans, C18:1:11trans, or the saturated C18:0 fatty acid and they were inactive.

Unsaturated cis fatty acids with shorter (C16:1:9cis) or longer (C20:1:11cis and C20:4:5,8,11,15 cis) carbon chains formed complexes that eluted after 1M NaCl, with yields comparable to C18:1:11cis, but lower than C18:1:9cis. The columns conditioned with the saturated fatty acids C6:0, C14:0 or C16:0 retained no apo-α-lactalbumin.

The results demonstrate that apo-α-lactalbumin interacts in a stereo-specific manner with C18:1 fatty acids, and that C18 fatty acids must be unsaturated and with the double bond in the cis conformation. Unsaturated fatty acids in the trans conformation were inactive, as were the saturated fatty acids. Furthermore the results confirmed that α-lactalbumin in its Ca^{2+} -bound form bound only to a very low degree to the C18:1 fatty acid conditioned column. The binding site for the unsaturated cis fatty acid that defines HAMLET is available only in the apo-conformation.

Example 2

Biological activity

Apoptosis induction was tested using the L1210 leukaemia cell line. Apoptosis induction in L1210 leukaemia cells exposed to the different protein-lipid complexes including the HAMLET control (M. Svensson, et al, (1999) *J Biol Chem*, **274**, 6388-96). HAMLET and the C18:1:11cis complex had killed 99-100% of the cells after six hours (Fig. 3 Table), but the other complexes had little or no effect on the cell viability. Both C18:1cis fatty acid protein complexes induced DNA fragmentation (b), but the C18:1trans fatty acid complexes were inactive. The C16 and C20 unsaturated fatty acid complexes caused an intermediate degree of DNA fragmentation, but no loss of cell viability.

L1210 cells were exposed to lipid extracts derived from HAMLET or from each of the other complexes. No effect on L1210 cell viability (Fig. 3 Table) or DNA fragmentation (c) was detected after six hours exposure to lipid concentrations corresponding to the amount present in 1.0 mg of protein, even though 0.3 mg of HAMLET was sufficient to kill the cells by apoptosis. At very high lipid concentrations, the cells died of necrosis but at no time were there evidence of apoptosis in response to lipids.

lipid complexes (Table in Fig. 3). Cell viability was reduced from 99% to 0% in six hours at a concentration of 0.3 mg/ml, and DNA fragmentation was observed. Interestingly, other C18:1:cis protein-fatty acid complexes had killed < 50% of the cells at this time (Table in Fig. 3). The C18:1:trans fatty acid complexes were inactive in the cellular assay, as were the C20 fatty acid complexes, and the C16 fatty acids complexes showed very low effects on cell viability.

The C18:1:cis fatty acid complexes had induced DNA fragmentation after six hours, suggesting that the cells were dying by apoptosis (Fig. 3a). We were surprised to find evidence of DNA fragmentation also in some cells exposed to the other fatty acid complexes, even though these cells remained viable at six hours.

These results demonstrate that the lipids do not trigger apoptosis, and that HAMLET is defined by both the protein and the lipid. They further demonstrate that only C18:1:9cis and C18:1:11cis, fulfil the criteria for a cofactor in the formation of HAMLET, even though some of the other fatty acids appeared to interact with apo-α-lactalbumin on the ion-exchange matrix.

Example 3

Structural correlates of the biologic activity

The ability to stabilise the protein in an apo-like conformation was determined by CD and ANS spectroscopy, and the structural integration was examined by NMR spectroscopy.

Conformation assessed by CD spectroscopy

The complexes eluting after 1M NaCl were examined by near UV CD spectroscopy (M. Svensson, et al., (2000) *Proc Natl Acad Sci* USA, **97**, 4221-6), using native or apo-α-lactalbumin and HAMLET as controls. The native α-lactalbumin control showed the characteristics of a well folded protein, with a minimum at 270 nm arising from tyrosine residues and a maximum at 294 nm arising from tryptophan residues. The apo-α-lactalbumin control had lost

most of the characteristic signals, indicating less restrained tyrosines and tryptophans. The C18:3:9,12,15cis, C18:3:6,9,12cis, C20:4:5,8,11,15cis and C18:1:6cis complexes resembled HAMLET with a loss of signal in the tyrosine and tryptophan regions, while remaining complexes were similar to the apo-α-lactalbumin control.

HAMLET was shown to resemble apo- α-lactalbumin, but seems to retain even less of the tertiary structure (Fig. 4a). The other eluted fatty acid-protein complexes showed two main spectral patterns. The C18:1:6cis, C18:3:9,12,15cis, C18:3,6,9,12cis and C20:4:5,8,11,15cis fatty acid complexes resembled HAMLET, while the C18:1:9trans, C18:1:11:cis or trans, C18:2:9,12cis and C16:1:9cis or trans complexes were identical to apo control (Fig. 4b-h). Unconverted apo-α-lactalbumin that eluted in the void was shown to revert to the native state in the presence of Ca^{2+}.

These results indicate that all fatty acids, which retain apo-α-lactalbumin on the column, stabilise the protein in a partially unfolded conformation.

Exposure of hydrophobic surfaces, as probed by ANS spectroscopy

Apo-α-lactalbumin is known to expose hydrophobic side chains, due to the mobility of the β-sheet. The complexes eluting after 1M NaCl were examined by ANS spectroscopy (M. Svensson, et al., (2000) *Proc Natl Acad Sci* USA, **97**, 4221-6) using native or apo-α-lactalbumin and HAMLET as controls. Native α-lactalbumin did not bind ANS as shown by the flat curve and the low signal at 490 nm, but apo-α-lactalbumin showed significant ANS binding with enhanced intensity and a maximum at 470 nm as expected from the increased hydrophobicity of this fold. All of the α-lactalbumin-fatty acid complexes except C20:1:11cis bound ANS. The C18:3:9,12,15cis, gammaC18:3:6,9,12cis and C18:1:6cis complexes [illegible]

The apo control showed the expected ANS binding with enhanced intensity and a maximum at 470 nm, while the native protein failed to bind ANS as shown by the flat curve and the low signal at 490 nm. HAMLET bound ANS with a blue shift of the curve, but the peak was lower than for apo-α-lactalbumin, (Fig. 5a).

The C18:1:6cis, C18:3:9,12,15cis, and C18:3:6,9,12cis fatty acid complexes, bound ANS with similar spectral intensity as HAMLET. Some other complexes (C18:1,9trans, C18:1,11cis and trans, C18:2,9,12cis, C16:1,9 cis and trans, C20:4,5,8,11,15cis) showed more intense ANS fluorescence than the apo-*a*-lactalbumin control. Finally, the C20:1:11cis complex did not bind any ANS (Fig. 5b-h).

To exclude the direct binding of ANS to the fatty acids in the protein-lipid complexes, mixtures of ANS to the fatty acid were subjected to spectroscopy. No ANS - fatty acid interaction was observed (data not shown).

These results demonstrated that the C18:1:9cis fatty acid complex retains the ability to interact with ANS, but the lower intensity suggested that the fatty acid in HAMLET might modify ANS binding. The negative results for C20:1:11cis complex suggested that the longer fatty acid hindered the interaction of hydrophobic surfaces in the protein with ANS.

<u>^{1}H-NMR spectroscopy</u>

^{1}H-NMR was used to resolve the structural basis for the difference in activity between the C18:1cis and the inactive protein-fatty acid complexes (Fig. 6).

Native α-lactalbumin showed the characteristics of a folded and well-ordered protein with narrow lines and significant shift dispersion, a large number of sharp signals in the aromatic region (around 7 ppm) and several out shifted methyl signals (between 0.7 and -0.6 ppm). The apo protein displayed narrow

lines and significant shift dispersion with significant variations relative to the native state in the chemical shifts of a large number of resonances. The aromatic and methylated regions are shown in the left and right panels, respectively (M. Svensson, et al., (2000) *Proc Natl Acad Sci USA*, **97**, 4221-6). HAMLET showed broad lines and lack of out shifted methyl signals suggestive of a partially unfolded state, and significantly different from the native protein. The spectrum obtained with the C18:1,11cis protein complex was virtually identical to HAMLET but the spectra of the trans fatty acid complexes showed more narrow lines and out shifted signals suggesting that the conformation of the C18:1:9cis or C18:1:11cis complexes are unique and that although the trans fatty acids bind to apo-α-lactalbumin, they do not alter the conformation so that HAMLET is formed.

The spectrum of both the C18:1,9cis and C18:1,11cis complexes showed broad lines and little shift dispersion. The lines in the aromatic region were clustered and there were no out shifted methyl signals below 0.7 ppm. The broad signals of the fatty acid suggested that they formed an integral part of the complex.

The trans isomer complexes (C18:1:9trans and C18:1:11trans) differed markedly from the C18:1:9cis or C18:1:11cis complexes. Signals from bound fatty acid were detected, but they were smaller than for the cis complexes. The protein lines were narrow, and out shifted both in the methyl and the aromatic regions. These data suggested that the trans fatty acids bind to apo-α-lactalbumin, but do not alter the conformation so that HAMLET is formed.

These results suggest that specific molecular interactions stabilise apo-α-lactalbumin in the HAMLET conformation, and only the unsaturated C18:1:9cis or C18:1:11cis fatty acids have the [illegible] to achieve this

Apo-α-lactalbumin differs from other known lipid-binding proteins in that it contains both α-helical and β-sheet domains. The intracellular lipid-binding protein family have an all β-barrel structure, forming a cavity which binds in a range of fatty acids varying in chain length and saturation (J. Thompson, et al., (1997) *J Biol Chem*, **272**, 7140-7150). Typically, the carboxylate head group of the fatty acids interacts with two to four positively charged amino acids, usually arginines, and the carbon chain is co-ordinated by six to ten hydrophobic amino acids. The crystal structure of human serum albumin has revealed six asymmetrically distributed, fatty acid binding sites within the repeating α-helical domain structure of the protein (S. Curry, et al., (1998) *Nature Struc Biol*, **5**, 827-835). Each hydrophobic pocket is capped at one end by basic or polar side chains, co-ordinating the fatty acid head group. While the binding of fatty acids to human serum albumin causes conformational changes with rotations of the three domains of the protein, and adjustments of side chains to make way for incoming fatty acid (S. Curry, et al., (1998) *Nature Struc Biol*, **5**, 827-835), the molecule does not unfold or change function. We may therefore conclude that the lipid cofactor function in the conversion of α-lactalbumin HAMLET differs both structurally and functionally from these previously known protein lipid interactions.

Tentative fatty acid binding sites were identified based on the three-dimensional structures of native apo-α-lactalbumin. The native α-lactalbumin molecule is a hydrophilic, acidic protein, exposing mainly charged and polar amino acids. Two hydrophobic regions are located in the interior of the globular structure. One is formed by residues from the C and D helices and the β-sheet domain in the interface between the two domains. The second is formed by residues in the A, B and 3_{10}- helices of the α-domain (Fig. 9) (L. C. Wu, et al., (1998) *J Mol Biol*, **280**, 175-82; M. Saito, (1999) *Protein engineering* **12**, 1097-1104). The crystal and NMR structures of bovine apo α-lactalbumin have

electrostatic interactions between its negatively charged head group and basic side-chains in the protein, as well as by van der Waal's contacts and hydrophobic effects with the tail that are optimized with the preferred stereo specific match (C18:1:9cis).

Example 4

Analysis of Variants of α-lactalbumin

The apo-conformation of α-lactalbumin is unstable and the protein reverts to the native state at neutral pH and at the Ca^{2+} concentrations present in the apoptosis assay. In the HAMLET complex, the protein maintains an apo-like conformation, however. As the lipid alone does not trigger apoptosis, it might act simply by stabilising the apo- conformation. A conformational change of the protein might then be sufficient to induce apoptosis, but the unstable nature of the apo conformation has precluded experiments testing the activity of the protein *per se*.

This question was addressed by site directed mutagenesis of the Ca^{2+} binding site. The bound Ca^{2+} ion is co-ordinated by a constellation of seven oxygen groups that form a distorted pentagonal bipyramid, but a mutation of the Aspartic acid residue at position 87 is sufficient to fully or partially inactivate the Ca^{2+} binding site, generating mutant proteins locked in the apo-conformation (Anderson P. J., et al., (1997) *Biochemistry* **36**, 11648-11654; Veprintsev D. B., et al., (1999) *Proteins* **37**, 65-72; Permyakov S. E., et al., (2001) *Proteins Eng* **14**, 785-789). This study examined if apoptosis can be triggered solely by a conformational change from the native to the apo-state. Furthermore, the importance of the Ca^{2+} binding site for the conversion to HAMLET was investigated.

Native human α-lactalbumin was purified from human milk by ammonium sulphate precipitation and phenyl sepharose chromatography as described (Svensson M., et al., (2000) *Proc*

in Tris[1] (10M Tris/HCI pH 8.5) by addition of 5 mM EDTA to remove bound Ca^{2+}. The conformational change was confirmed by near UV CD and ANS spectroscopy. Bovine α-lactalbumin was purchased from Sigma, St. Louis, MO, USA, and used without further purification.

In addition, a mutated bovine protein (D87A and D87N) was expressed in *E. coli*, purified, folded and lyophilised as described (Anderson P. J., et al., (1997) *Biochemistry* **36**, 11648-11654; Permyakov S. E., et al., (2001) *Proteins Eng* **14**, 785-789).

A column (14 cm x 1.6 cm) packed with DEAE-Trisacryl M (BioSepra, France) was attached to a Bio-Logic chromatography system (Bio-Rad Laboratories, Hercules, CA), and eluted with a NaCl gradient (buffer A: 10 mM Tris/HCI pH 8.5; buffer B: buffer A containing 1 M NaCl). The matrix was conditioned with oleic acid (Larodan biochemicals, Malmo, Sweden). Ten milligrams was dissolved in 500 µl 99.5% ethanol by sonication (3 minutes using a Branson 2200 bath sonicator, Branson, Danbury, USA). After addition of 10 ml of 10 mM Tris/HCl, pH 8.5, the lipid solution was applied to a newly packed DEAE-Trisacryl M matrix and dispersed through out the matrix using a NaCl gradient.

Ten mg of each of human and recombinant and native bovine α-lactalbumin was dissolved in 10 ml of 10 mM Tris/HCl pH 8.5 and added to the column. The protein fraction eluting after high salt was desalted by dialysis (Spectra/Pore, Spectrum Medical Industries, Laguna Hills, CA, membrane cut off 3.5 kDa) against distilled water with at least four changes of water in 100-fold volume excess, and then lyophilised.

The products were then subjected to spectroscopic analysis. The proteins or protein fractions were dialyzed against doubly distilled water and lyophilised. Stock solutions were prepared by dissolving the lyophilised material in 10 mM potassium phosphate buffer at pH 7.5, and concentrations determined as the absorbance at 280 nm (A/1=C (mg/ml) where 1 mg/ml of α-

lactalbumin is 70μM). The spectra were recorded at the appropriate dilution.

Circular Dichroism (CD) spectra were obtained on a JASCO J-720 spectro-polarimeter with a JASCO PTC-343 Peltier type thermostated cell holder. Quartz cuvettes were used with 1 cm path length and spectra were recorded at 25 °C between 240 and 320 nm. The wavelength step was 1 nm, the response time δ s and the scan rate was 10 nm per minute. Six scans were recorded and averaged for each spectrum. Baseline spectra were recorded with pure buffer in the cuvette and substracted from the protein spectra.

The mean residue ellipticity q_m (mdeg x cm^2 x $dmol^{-1}$) was calculated from the recorded ellipticity, q, as

$$q_m = q / (c \cdot n \cdot 1)$$

where c is the protein concentration in M, n the number of residues in the protein (123 in this case), 1 the path length in nm and q is the ellipticy in degrees.

Fluorescence spectra were recorded at 25°C on a Perkin Elmer LS-50B spectrometer using a quartz cuvette with 1 cm excitation path length. Intrinsic (tryptophan) fluorescence emission spectra were recorded between 305 and 530 nm (step 1 nm) with excitation at 295 nm. The excitation bandwidth was 3 nm and the emission was 5 nm. ANS fluorescence emission spectra were recorded at 25°C on a Perkin Elmer LS-50B spectrometer using a quartz cuvette with 1 cm excitation path length, between 400 and 600 nm (step 1 nm) with excitation at 385 nm. Both the excitation and emission bandpass were set to 5 nm. ANS ammonium salt (Fluka, Buchs, Switzerland) was added stepwise and the spectra at 1.5 molar equivalents are shown.

The results are illustrated in Figure 7. Panel A shows ribbon diagrams of the calcium-binding loop with the co-ordinating side chains shown as darkly shaded lines. In the wild-type protein calcium is co-ordinated by K79, D82,84,87 (arrow) and D88. If D87 is changed to A (arrow), the protein looses its ability to bind calcium. If D87 is changed to N (arrow) the protein can still bind calcium but with low affinity.

The results of the investigation into the tertiary structure of the two mutants is shown in panel B. Spectra were recorded in sodium phosphate buffer without EDTA. Native bovine α-lactalbumin had a minimum at 270nm arising from tyrosine residues and a maximum at 294 nm arising from tryptophan residues. Apo α-lactalbumin showed the characteristic loss of signal, indicating less restrained tyrosines and tryptophans. The D87A mutant showed an almost complete loss of ellipticity consistent with a partially unfolded conformation. The spectrum of the D87N mutant showed decreased ellipticity in the tyrosine and tryptophan regions, although not to the same extent as the D87A mutant.

The result of intrinsic fluorescence spectroscopy is shown in panel C. Native bovine α-lactalbumin showed an intensity maximum at 335 nm and a shoulder at 320 nm, indicative of tryptophan residues in a folded hydrophobic core, but shifted to 350 in apo α-lactalbumin indicating that the tryptophans are more accessible to the solvent. Both the D87A and the D87N mutant showed intensity maxima at 350 nm resembling apo-α-lactalbumin.

Fluorescence spectra at 1.5 equivalents of ANS are shown in Figure 7 panel D. Native bovine α-lactalbumin did not bind ANS but resembled ANS added to pure buffer. Apo α-lactalbumin bound ANS with a maximum at 475 nm and significantly enhanced intensity. The D87N and D87A mutants bound ANS with intensity maxima at 475 nm, strongly resembling the spectrum of the apo control.

with 10% fetal calf serum, non essential amino acids, sodium pyruvate and 50 µg gentamicin/ml, Life Technologies, Gibco BRL, Paisly, United Kingdom) and seeded into 24 well plates (Falcon, Becton Dickinson, New Jersey, USA) at a density of 2 x 10^6/well. The different agonists were dissolved in cell culture medium, without fetal calf serum, and added to the cells (final volume 1 ml per well). Plates were incubated at 37°C in 5% CO_2 atmosphere and 100 µl of fetal calf serum was added to each well after 30 minutes. Cell culture medium served as a control.

Cell viability was determined by Trypan blue exclusion after six hours of incubation. For analysis, 30 µl of the cell suspension was mixed with 30 µl of a 0.2% trypan blue solution and the number of stained cells (dead cells) per 100 cells was determined by interference contrast microscopy (Ortolux II, Leitz Wetzlar, Germany).

DNA fragmentation

Oligonucleosome length DNA fragments were detected by agarose gel electrophoresis. The cell suspension remaining after trypan blue (970 µl, 2x10^6/ml) was lysed in 5 mM Tris, 20 mM EDTA, 0.5% Triton X-100 pH 8.0 at 4°C for 1 hour and centrifuged at 13,000 x g for 15 minutes. DNA was ethanol precipitated over night in -20°C, treated with proteinase K and RNAse, loaded on 1.8% agarose gels and electrophoresed with constant voltage set at 50V over night. DNA fragments were visualised with ethidium bromide using a 305nm UV-light source and photographed using Polaroid type 55 positive-negative film.

Mutant Proteins

The ability of the mutant proteins to induce apoptosis was tested using the L1210 cell line. The proteins were suspended in cell culture medium at 2 mg / ml and the cell viability was determined after six hours of incubation as was the DNA fragmentation. The [illegible] induced apoptosis to [illegible] but the mutant

Figure 8, Panel A Table 1 shows the viability of L1210 cells after 6 hours' exposure to the mutant proteins. The mutants were unable to kill the cells even at a concentration of 1.0 mg/ml (cf the results for BAMLET (see Fig. 9) where the viability reduced from 98% to 4%.

The mutant proteins did not induce DNA fragmentation, but BAMLET stimulated the formation of the characteristic DNA ladder as shown in panel B.

These results demonstrate that the protein without associated oleic acid is not sufficient to induce apoptosis in tumour cells.

Example 6

Preparation of BAMLET (Bovine a-lactalbumin made lethal to tumour cells)

In view of the structural homology between the human and bovine proteins it should be possible to convert bovine a-lactalbumin to HAMLET like molecule, with apoptosis inducing properties. Hence, bovine a-lactalbumin was subjected to the conversion conditions previously used for human a-lactalbumin. Bovine α-lactalbumin was treated with EDTA to remove Ca^{2+}, subjected to ion exchange chromatography on a C18:1 conditioned column and eluted with a NaCl gradient. A large portion of the applied material eluted in the void but about 40% formed a sharp peak after 1M NaCl (arrow Figure 9a). The eluate after high salt was saved for analysis. Human α-lactalbumin was converted with higher efficiency.

A large proportion of the applied material eluted in the void (about 60%), but a small sharp peak eluted after 1M NaCl (Fig. 9a).

The apoptosis-inducing activity of the high salt peak, named BAMLET, was investigated using the L1210 mouse leukemia cell line as described above. Loss of cell viability and DNA fragmentation were used as end points. The L1210 cells died rapidly when

exposed to HAMLET (0.3 mg/ml) and DNA fragmentation was induced. The L1210 cells were equally sensitive to BAMLET.

BAMLET reduced cell viability from 99% to 12% at 0.3 mg / ml, after six hours incubation and induces DNA fragmentation (Fig.9b). There was no apparent difference in efficiency of apoptosis induction between HAMLET and the bovine equivalent (Fig.9b).

The tertiary structure of BAMLET was assessed using near UV CD spectroscopy. Native bovine a-lactalbumin shared the characteristic spectrum of a well-folded protein with tyrosine dip and tryptophan peak, and native bovine a-lactalbumin did not bind ANS. The bovine apo protein had a reduced signal in both the tyrosine and tryptophan regions, indicative of a partially folded protein with flexible side chains, and significant ANS binding with the maximum at 470nm and enhanced intensity. The bovine complex strongly resembled both HAMLET and the apo-control (Fig.9c). The native and apo controls were as in Fig. 7. HAMLET showed decreased ellipticity in the tyrosine and tryptophan regions characteristics of a partially unfolded protein. BAMLET had spectra similar to the apo control and to HAMLET, indicating flexible aromates.

The intrinsic fluorescence spectrum native bovine a-lactalbumin had an intensity maximum at 320 nm, as expected from tyrptophan residues in the folded protein. The apo- protein had an intensity-maximum at 345 nm and a shoulder at 360 nm, indicating that the tyrptophans are more exposed (Fig. 9e). HAMLET showed an intrinsic fluorescence intensity maximum at 345 nm and a shoulder at 360 nm indicating solvent exposed tryptophans. The spectrum of BAMLET was similar to that of HAMLET but without the shoulder. The results indicate that tryptophan residues are shielded from solvent in the native protein, but are more solvent exposed in the [illegible] HAMLET [illegible]

Spectroscopic characterisation of the D87A- and D87N-BAMLET

The conformations of D87A and D87N-BAMLET were compared to native and apo-bovine a-lactalbumin and to BAMLET. Near UV CD spectroscopy was carried out on the complexes, with the native, apo and BAMLET controls as in Figs. 7 and 9. The D87A-BAMLET spectrum was very similar to the unconverted D87A protein with virtually no ellipiticy showing that D87A-BAMLET is in the apo configuration. The spectrum of D87N-BAMLET was virtually identical to that of BAMLET with reduced ellipiticy in both the tyrosine and tryptophan region (Fig.10d).

Intrinsic tryptophan fluorescence spectroscopy was conducted with the native, apo-α-lactalbumin and BAMLET controls as in figures 1 and 3. The results (Fig. 10e) with D87A- and D87N-BAMLET showed intensity maxima at 345 nm with shoulder at 355 nm strongly resembling BAMLET and the human apo-a-lactalbumin control, suggesting that tryptophans are accessible to solvent.

ANS spectroscopy was conducted, with the native, apo- and BAMLET controls as in Figures 7 and 9. Both D87A- and D87N-BAMLET bound ANS, with spectra resembling BAMLET and the apo-α-lactalbumin control. D87A-D87N-BAMLET bound ANS with the intensity maximum shifted to 470 nm and an increased quantum yield compared to the native control, indicating exposed hydrophobic surfaces in all proteins (Fig. 10f).

These results demonstrated that D87A- and D87N-BAMLET maintain the partially folded state with structural and functional properties resembling HAMLET. Calcium removal prior to oleic acid treatment was not required for the D87A mutant because the protein is most likely free from bound calcium and largely rests in the apo form.

As the proteins maintained their biologic activity, it appears that a functional calcium-binding site is not required for the

conversion of a-lactalbumin to an apoptosis-associated conformation.

Claims

1. A biologically active complex comprising
(i) a cis C18:1:9 or C18:1:11 fatty acid or a different fatty acid with a similar configuration; and
(ii) α-lactalbumin from which calcium ions have been removed, or a variant of α-lactalbumin from which calcium ions have been removed or which does not have a functional calcium binding site; or a fragment of either of any of these, provided that any fragment comprises a region corresponding to the region of α-lactalbumin which forms the interface between the alpha and beta domains, and further provided that when (ii) is alphalactalbumin, (i) is other than C18:1:9 cis fatty acid.

2. A complex according to claim 1 which includes a variant of α-lactalbumin in
which the calcium binding site has been modified so that the affinity for calcium is reduced, or it is no longer functional.

3. A complex according to claim 2 wherein the variant as a mutation at one of the K79, D82, D84, D87 and D88.

4. A complex according to claim 3 wherein the modification is at D87 which includes a variant of α-lactalbumin having a D87A or D87N variants.

5. A complex according to any one of the preceding claims which comprises a fragment of α-lactalbumin or a variant thereof, and where the fragment includes the entire region from amino acid 34-86 of the native protein.

6. A complex according to any one of the preceding claims wherein the α-lactalbumin is human or bovine α-lactalbumin or a variant of either of these.

7. A complex according to claim 6 wherein the α-lactalbumin is human α-lactalbumin.

8. A complex according to claim 6 wherein the α-lactalbumin is mutant bovine α-lactalbumin which includes an S70R mutation.

9. A pharmaceutical composition comprising a complex according to any one of the preceding claims in combination with a pharmaceutically acceptable carrier.

10. A method for treating cancer which comprises administering to cancer cells a complex according to any one of claims 1 to 8 or a composition according to claim 9.

11. A method for treating bacterial infections which comprises administering to a patient in need thereof, a complex or a composition as described above.

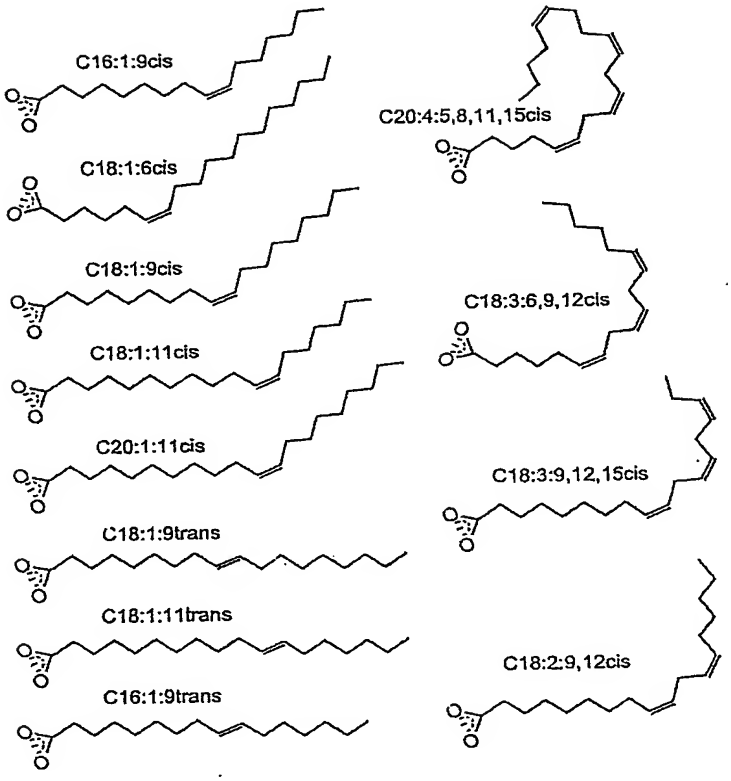
Abstract

Biologically Active Complex

A biologically active complex comprising
(i) a cis C18:1:9 or C18:1:11 fatty acid or a different fatty acid with a similar configuration; and
(ii) α-lactalbumin from which calcium ions have been removed, or a variant of α-lactalbumin from which calcium ions have been removed or which does not have a functional calcium binding site; or a fragment of either of any of these, provided that any fragment comprises a region corresponding to the region of α-lactalbumin which forms the interface between the alpha and beta domains, and further provided that when (ii) is alphalactalbumin, (i) is other than C18:1:9 cis fatty acid.

These complexes have therpeutic applications for example in the treatment of cancer and as antibacterial agents.

Figure 1


C16:1:9cis
C18:1:6cis
C18:1:9cis
C18:1:11cis
C20:1:11cis
C18:1:9trans
C18:1:11trans
C16:1:9trans
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C18:3:6,9,12cis
C18:3:9,12,15cis
C18:2:9,12cis

Figure 2

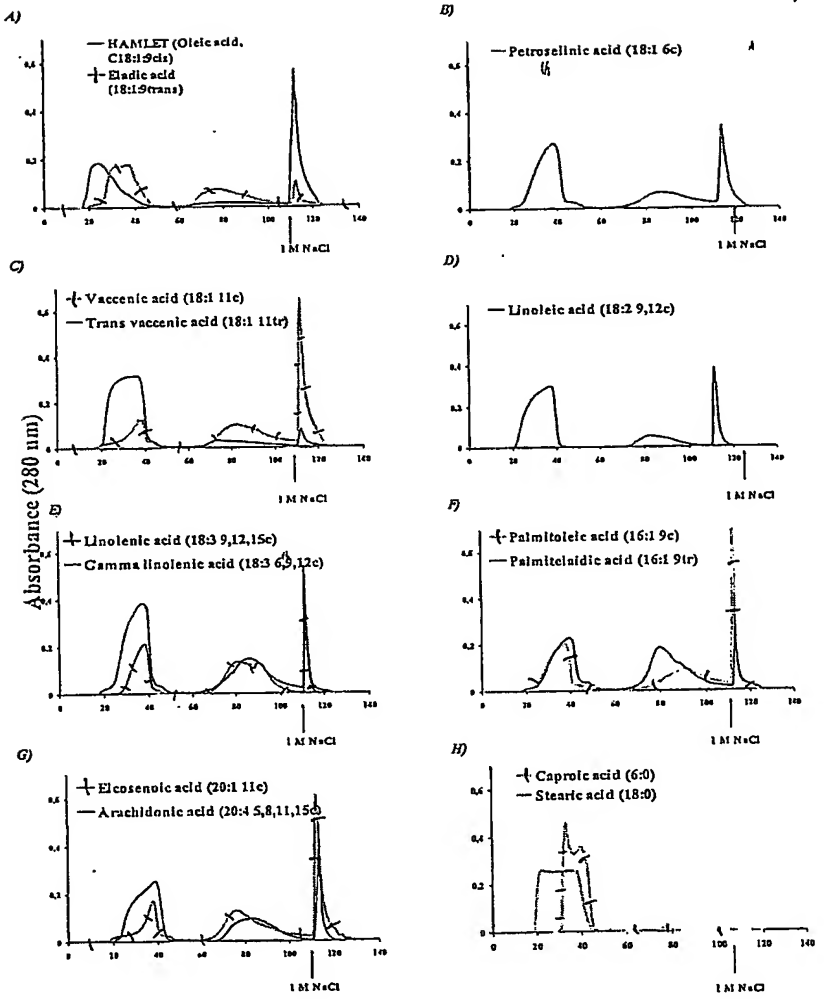

A)
— HAMLET (Oleic acid, C18:1 9cis)
+ Elaidic acid (18:1 9trans)
1 M NaCl
B)
— Petroselinic acid (18:1 6c)
1 M NaCl
C)
+ Vaccenic acid (18:1 11c)
— Trans vaccenic acid (18:1 11tr)
1 M NaCl
D)
— Linoleic acid (18:2 9,12c)
1 M NaCl
E)
+ Linolenic acid (18:3 9,12,15c)
— Gamma linolenic acid (18:3 6,9,12c)
1 M NaCl
F)
+ Palmitoleic acid (16:1 9c)
— Palmitelaidic acid (16:1 9tr)
1 M NaCl
G)
+ Eicosenoic acid (20:1 11c)
— Arachidonic acid (20:4 5,8,11,15c)
1 M NaCl
H)
+ Caproic acid (6:0)
— Stearic acid (18:0)
1 M NaCl
Absorbance (280 nm)
Time (min)

Figure 3

a) The viability of L1210 cells after exposure to converted material and free fatty acids

	Viability (%)
Medium control	99
Fatty acid/protein complexes:	
18:1: 9c (HAMLET)	0
18:1: 9tr	98
18:1:11c	1
18:1:6c	60
18:3 c	32
γ18:3 c	43
18:1 :11tr	98
18:2 c	89
16:1:9c	73
16:1 :9tr	98
20:1:11c	96
20:4 c	96
Fatty acids:	
18:1:9c	99
18:1: 9tr	98
18:1:6c	97
18:1: 11tr	96
18:2 c	98
18:3 c	99
γ18:3 c	99
16:1:9c	96
16:1:9tr	98
20:1:11c	99
20:4 c	98

Figure 4

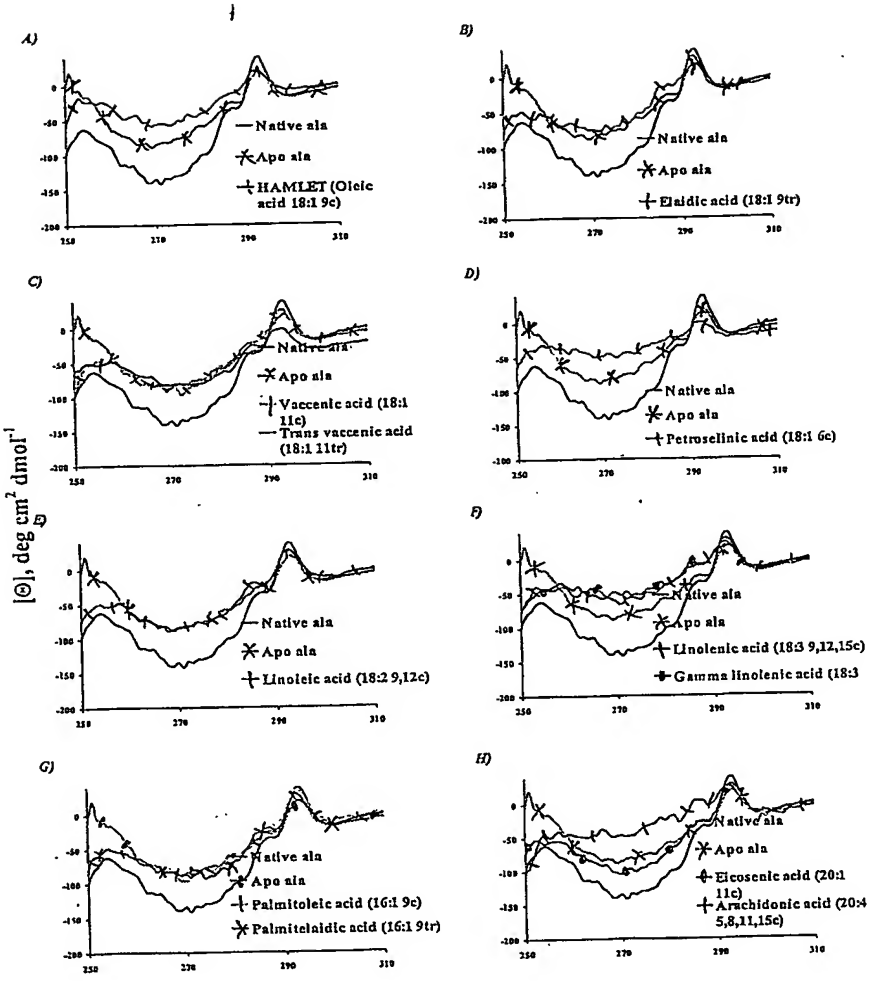

A)
Native ala
Apo ala
HAMLET (Oleic acid 18:1 9c)
B)
Native ala
Apo ala
Elaidic acid (18:1 9tr)
C)
Native ala
Apo ala
Vaccenic acid (18:1 11c)
Trans vaccenic acid (18:1 11tr)
D)
Native ala
Apo ala
Petroselinic acid (18:1 6c)
E)
Native ala
Apo ala
Linoleic acid (18:2 9,12c)
F)
Native ala
Apo ala
Linolenic acid (18:3 9,12,15c)
Gamma linolenic acid (18:3
G)
Native ala
Apo ala
Palmitoleic acid (16:1 9c)
Palmitelaidic acid (16:1 9tr)
H)
Native ala
Apo ala
Eicosenic acid (20:1 11c)
Arachidonic acid (20:4 5,8,11,15c)
[Θ], deg cm^2 dmol^{-1}
Wavelength (nm)

Figure 5

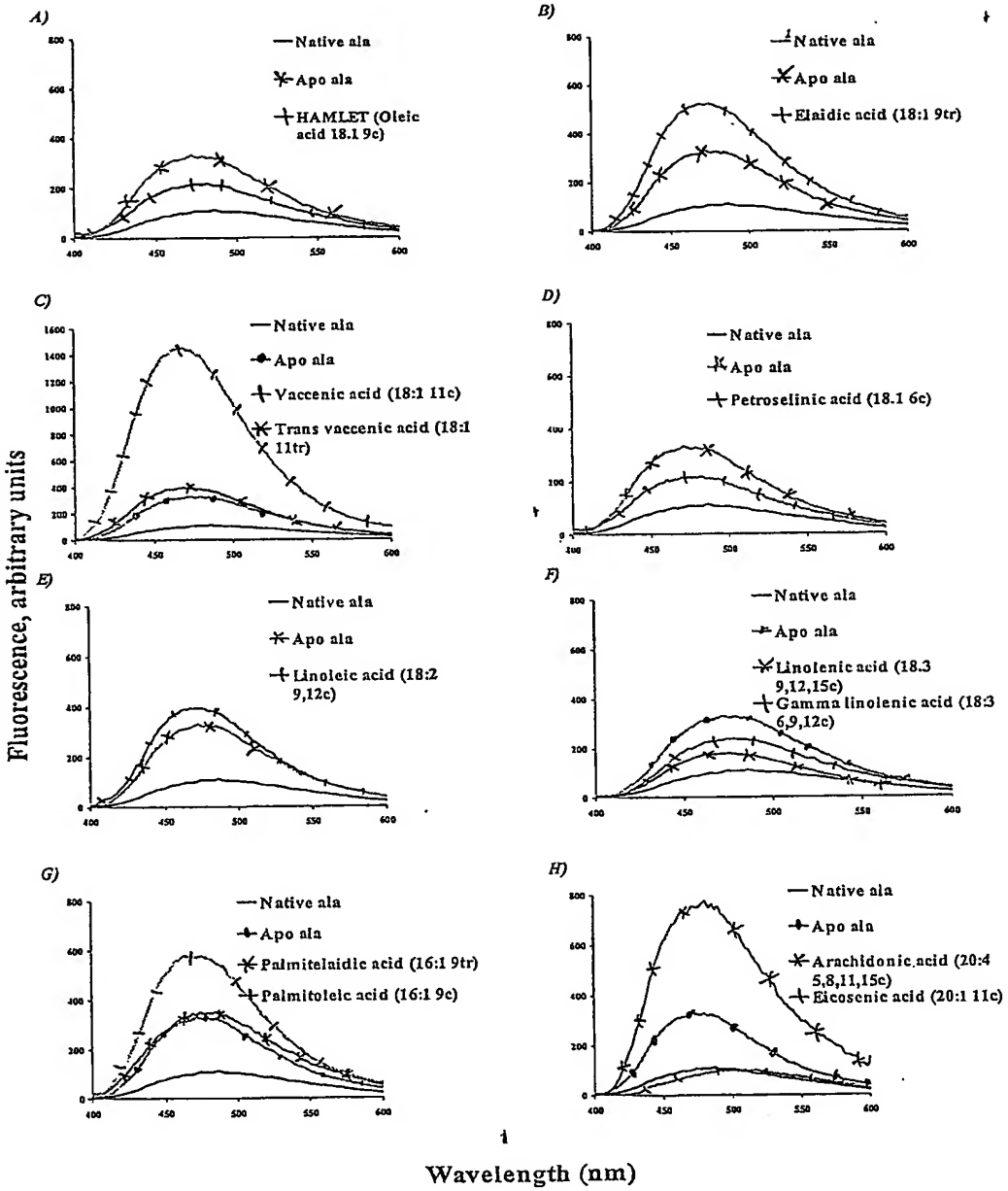
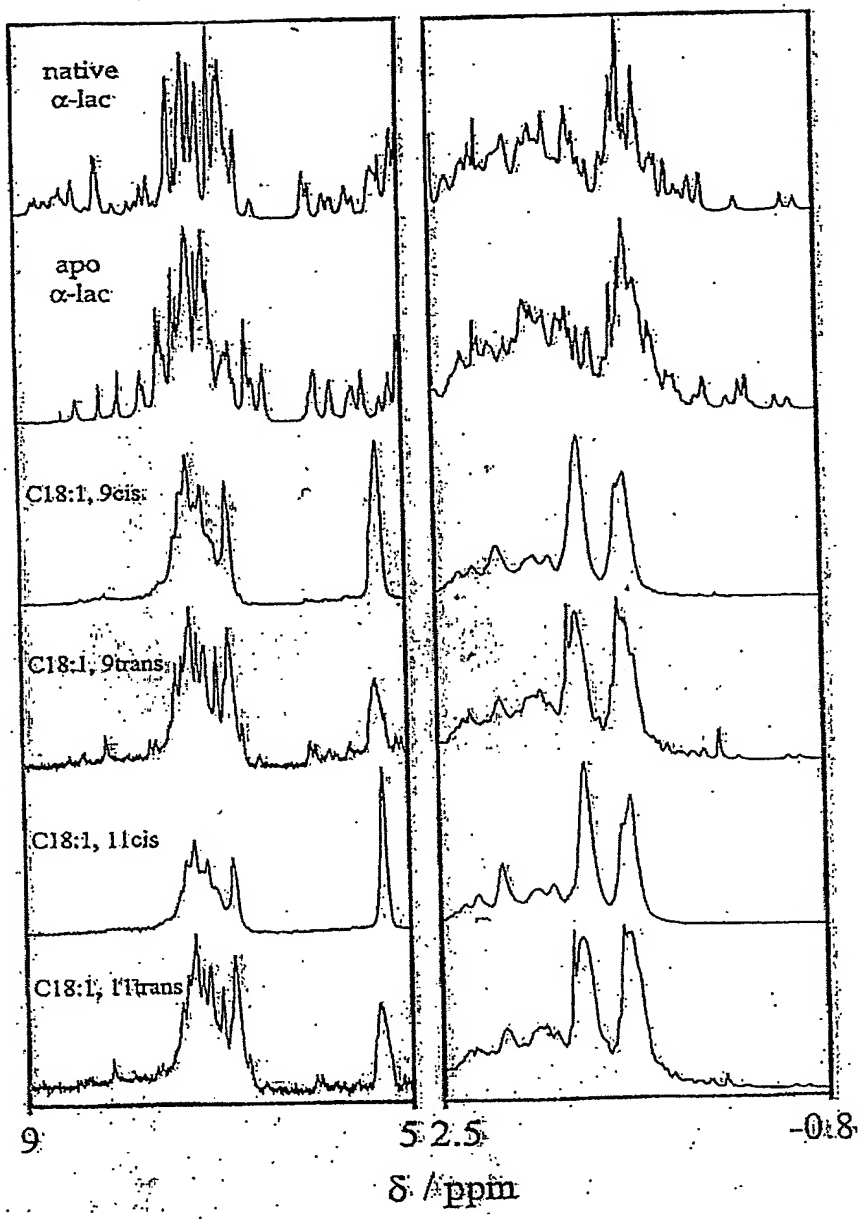
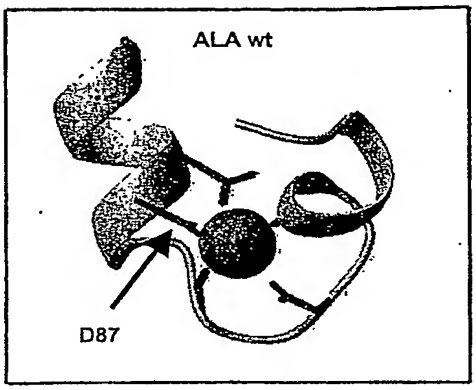

A)
Native ala
Apo ala
HAMLET (Oleic acid 18.1 9c)
B)
Native ala
Apo ala
Elaidic acid (18:1 9tr)
C)
Native ala
Apo ala
Vaccenic acid (18:1 11c)
Trans vaccenic acid (18:1 11tr)
D)
Native ala
Apo ala
Petroselinic acid (18.1 6c)
E)
Native ala
Apo ala
Linoleic acid (18:2 9,12c)
F)
Native ala
Apo ala
Linolenic acid (18.3 9,12,15c)
Gamma linolenic acid (18:3 6,9,12c)
G)
Native ala
Apo ala
Palmitelaidic acid (16:1 9tr)
Palmitoleic acid (16:1 9c)
H)
Native ala
Apo ala
Arachidonic acid (20:4 5,8,11,15c)
Eicosenic acid (20:1 11c)
Fluorescence, arbitrary units
Wavelength (nm)

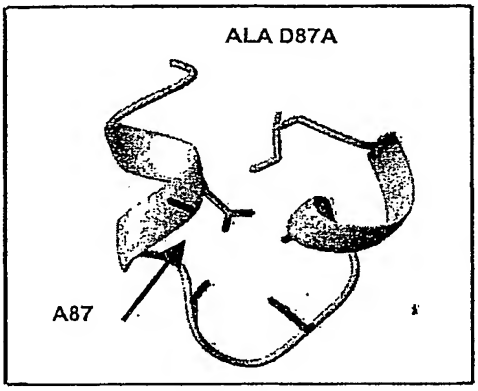
Figure 6

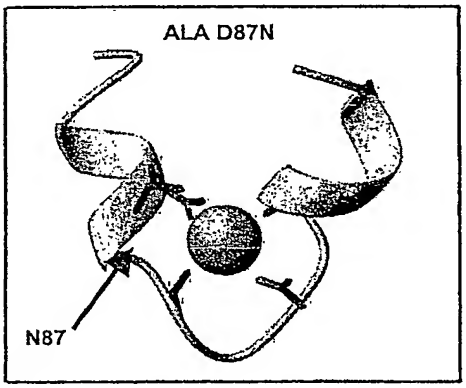

native
α-lac
apo
α-lac
C18:1, 9cis
C18:1, 9trans
C18:1, 11cis
C18:1, 11trans
9
5
2.5
-0.8
δ /ppm

gure 7

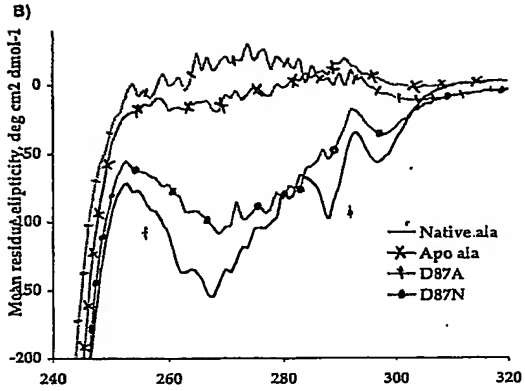
A)


ALA wt
D87

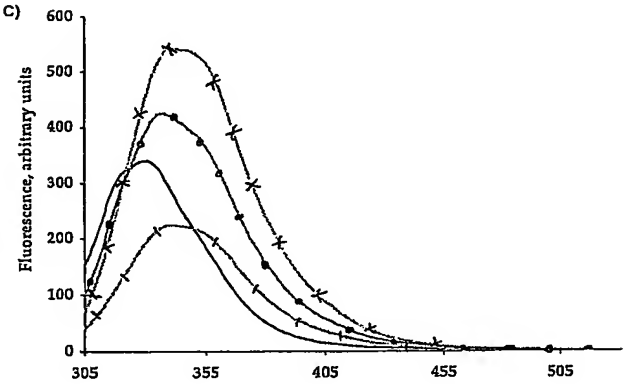

ALA D87A
A87


ALA D87N
N87

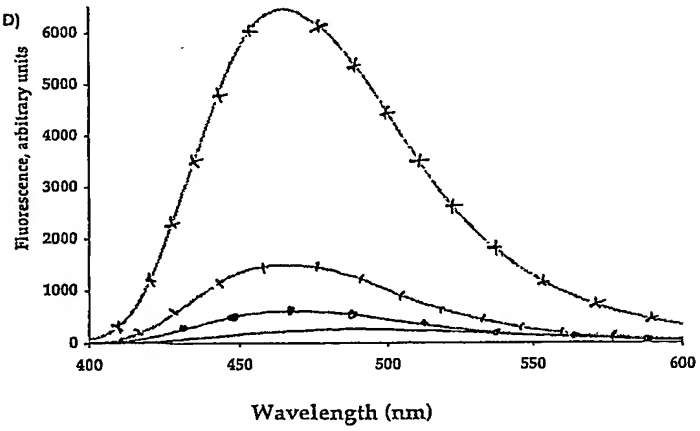
B)


Mean residue ellipticity, deg cm2 dmol-1
0
-50
-100
-150
-200
240
260
280
300
320
Native.ala
Apo ala
D87A
D87N

C)


Fluorescence, arbitrary units
600
500
400
300
200
100
0
305
355
405
455
505

D)


Fluorescence, arbitrary units
6000
5000
4000
3000
2000
1000
0
400
450
500
550
600
Wavelength (nm)

A)

Table I. The viability of L1210 cells after treatment with various forms of α-lactalbumin.

	Cell viability (%)
Medium control	98
α-lactalbumin*:	
native	99
D87A	99
D87N	97
HAMLET†	4

* the concentration was 1.0 mg/ml
† the concentration was 0.3 mg/ml

B)

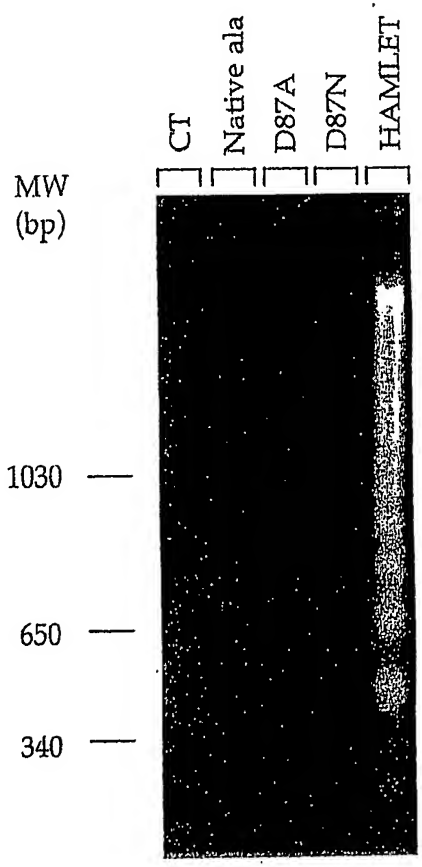
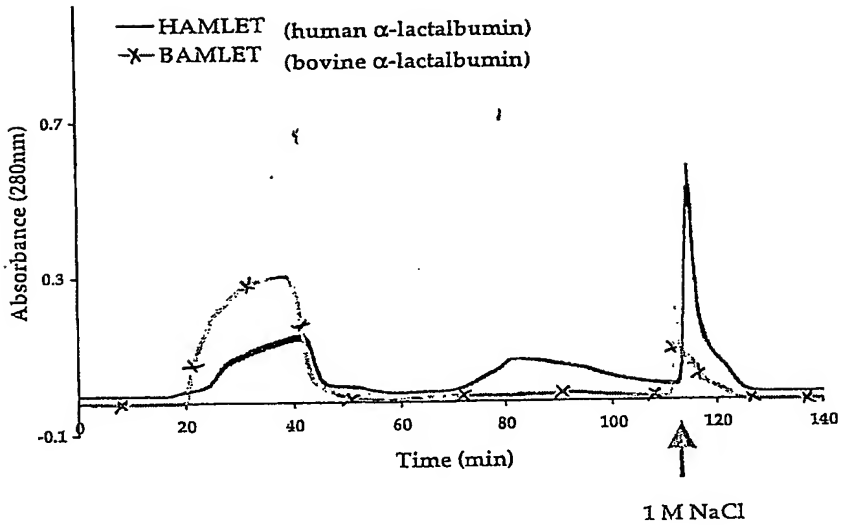


figure 8

Figure 9

A)



B)

Table II. Viability of L1210 cells after treatment with BAMLET and HAMLET.

	Cell viability (%)
Medium control	98
HAMLET	
0.2 mg/ml	67
0.3 mg/ml	9
BAMLET	
0.2 mg/ml	76
0.3 mg/ml	8

C)

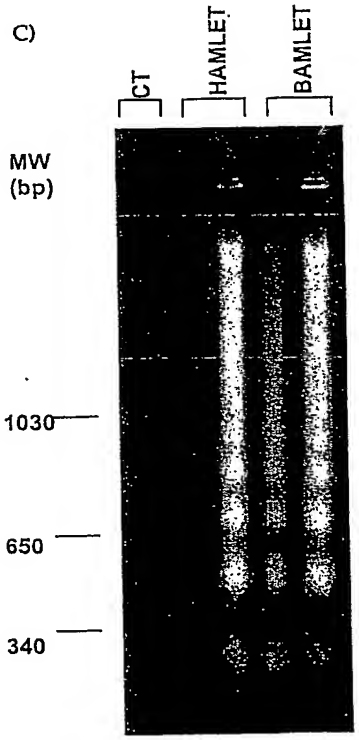
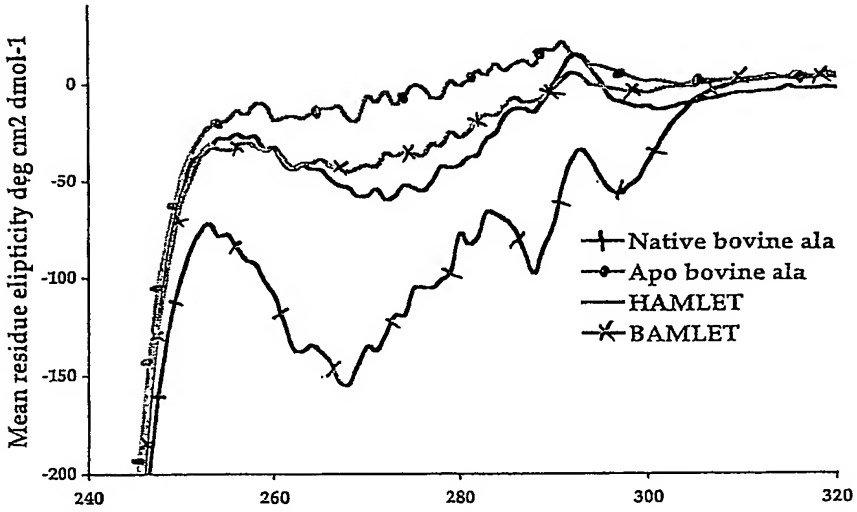
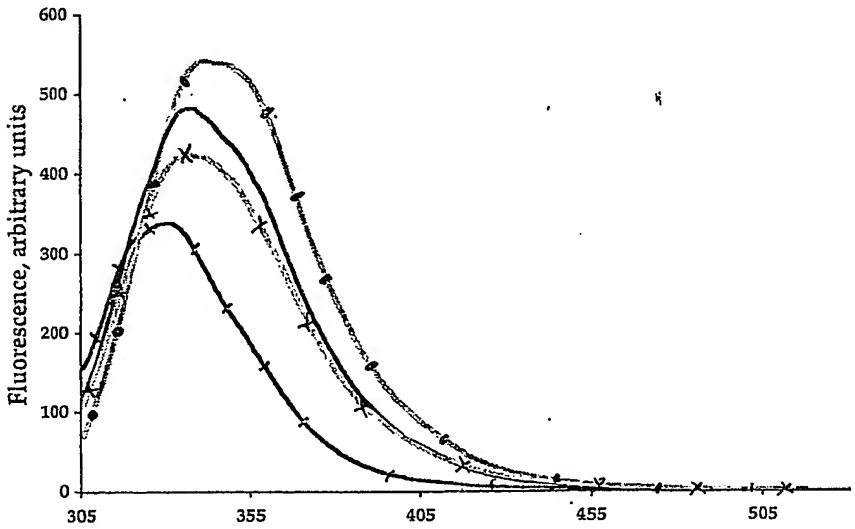


Figure 9 cont.

D)



E)



F)

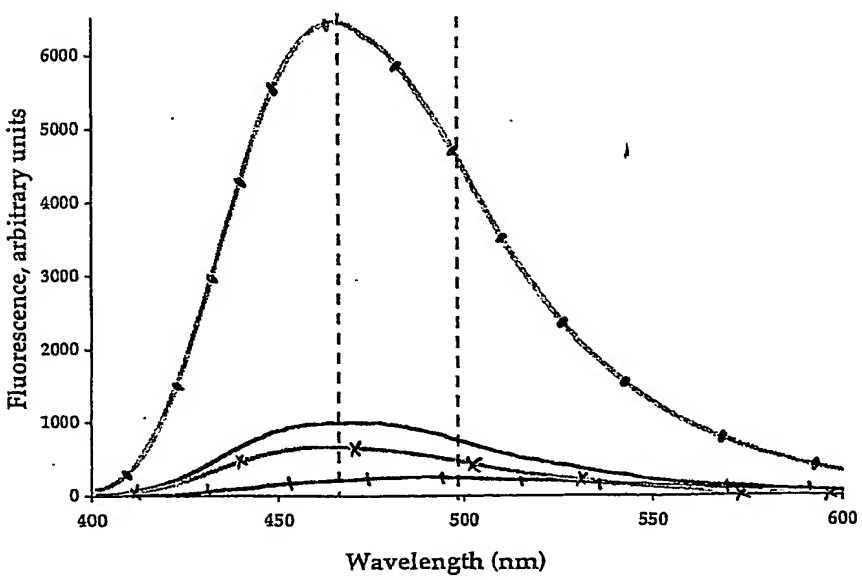
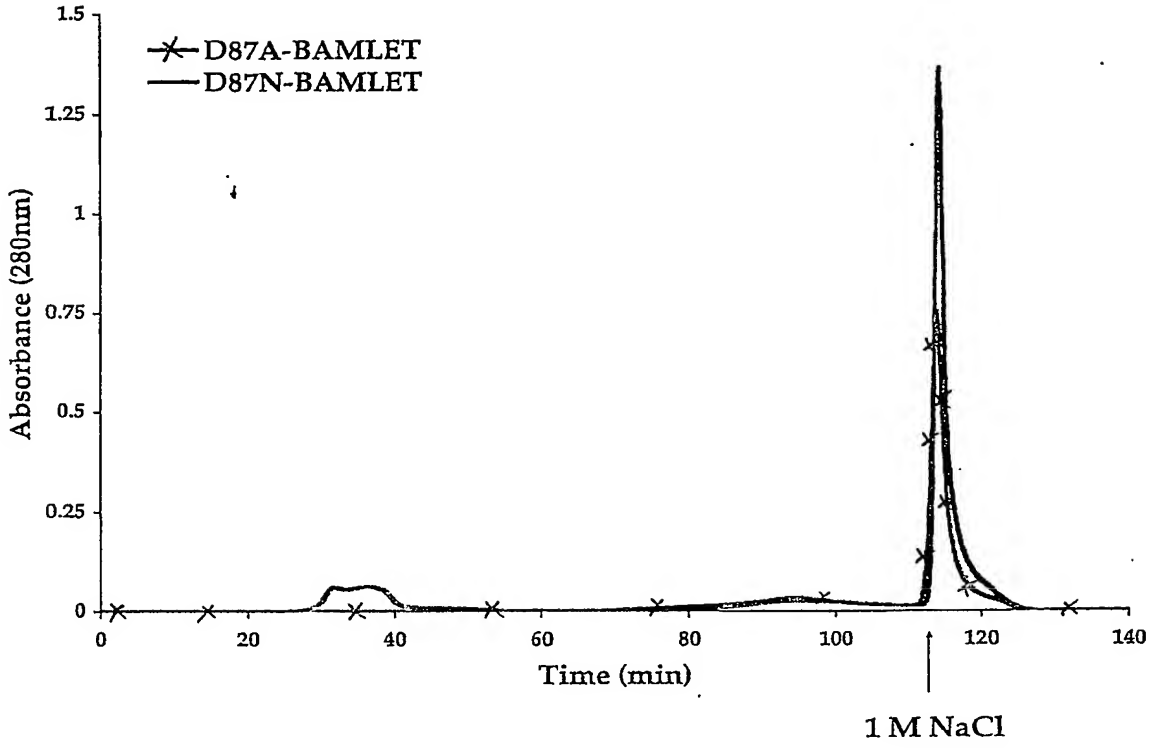


Figure 10

A)



B)

Table III. The viability of L1210 cells after treatment with D87A- and D87N-BAMLET.

	Cell viability (%)
Medium control	98
α-lactalbumin*	
native	97
D87A-BAMLET	13
D87N-BAMLET	17
BAMLET†	0

*the concentration was 0.5 mg/ml
†the concentration was 0.3 mg/ml

C)

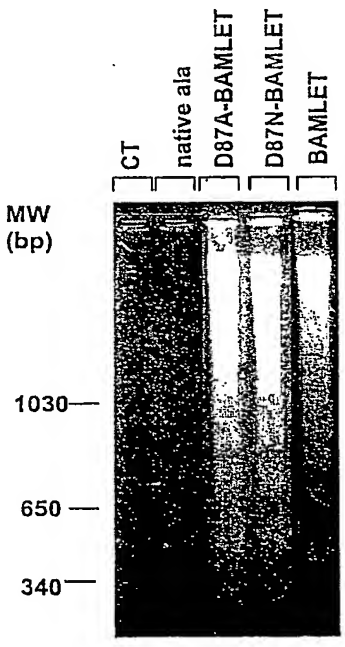
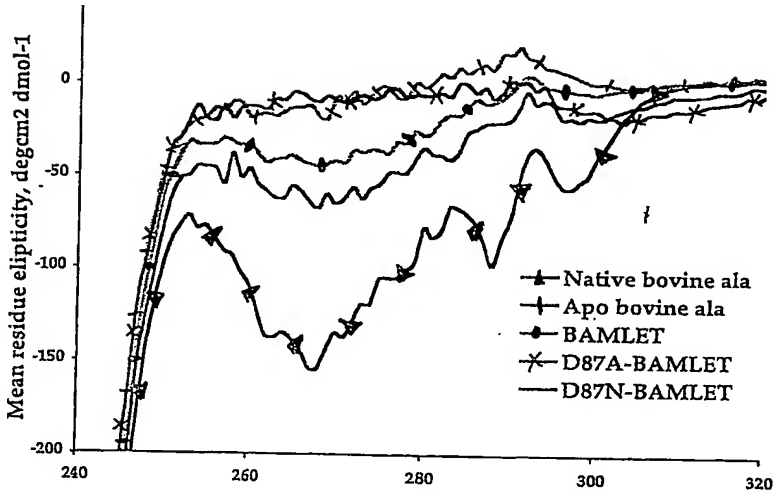
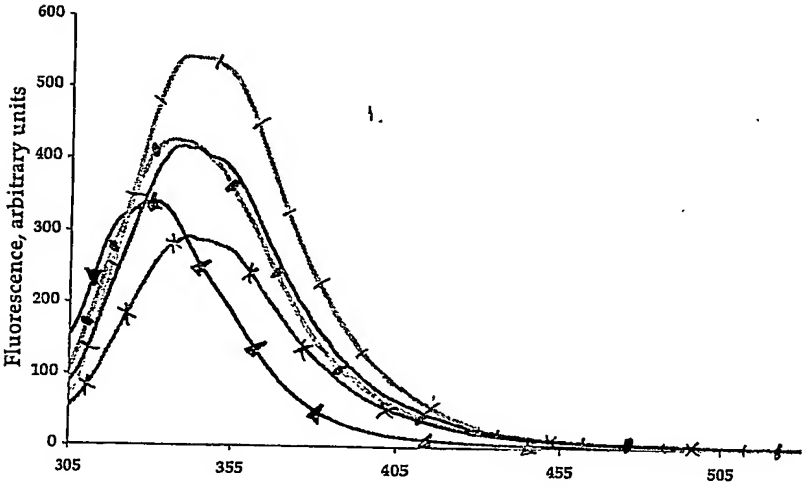


Figure 10 cont.

D)



E)



F)